

# Taking advantage of an old concept, “illegitimate transcription”, for a proposed novel method of genetic diagnosis of McArdle disease

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**Purpose:** McArdle disease is a metabolic disorder caused by pathogenic mutations in the *PYGM* gene. Timely diagnosis can sometimes be difficult with direct genomic analysis, which requires additional studies of cDNA from muscle transcripts. Although the “nonsense-mediated mRNA decay” (NMD) eliminates tissue-specific aberrant transcripts, there is some residual transcription of tissue-specific genes in virtually all cells, such as peripheral blood mononuclear cells (PBMCs).

**Methods:** We studied a subset of the main types of *PYGM* mutations (deletions, missense, nonsense, silent, or splicing mutations) in cDNA from easily accessible cells (PBMCs) in 12 McArdle patients.

**Results:** Analysis of cDNA from PBMCs allowed detection of all mutations. Importantly, the effects of mutations with unknown

pathogenicity (silent and splicing mutations) were characterized in PBMCs. Because the NMD mechanism does not seem to operate in nonspecific cells, PBMCs were more suitable than muscle biopsies for detecting the pathogenicity of some *PYGM* mutations, notably the silent mutation c.645G>A (p.K215=), whose effect in the splicing of intron 6 was unnoticed in previous muscle transcriptomic studies.

**Conclusion:** We propose considering the use of PBMCs for detecting mutations that are thought to cause McArdle disease, particularly for studying their actual pathogenicity.

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**Key Words:** diagnosis; glycogenosis type V; McArdle disease; *PYGM* mutations; transcript analysis

## INTRODUCTION

Glycogenosis type V (glycogen storage disease type V, McArdle disease, or myophosphorylase deficiency; OMIM database number 232600) is an autosomal recessive disorder of muscle glycogen metabolism. Patients harbor pathogenic mutations in both alleles of the phosphorylase, glycogen, muscle (*PYGM*) gene (MIM 608455), which encodes the muscle-specific isoform of glycogen phosphorylase (or “myophosphorylase”).<sup>1</sup> The clinical presentation in adults is dominated by exercise intolerance,<sup>2</sup> i.e., acute crises of early fatigue and myalgia, which are frequently accompanied by rhabdomyolysis, as reflected by marked increases in serum levels of creatine kinase or myoglobinuria.<sup>3</sup> Very young patients also show elevated serum creatine kinase and the abovementioned “crises,” notably during physical education classes or when playing on the school playground, whereas preschool children may be late in learning to walk and

“bottom shuffle” more often than they crawl.<sup>4</sup> These children may also seek to be carried more frequently.<sup>4</sup>

The current best diagnostic tool for McArdle disease is genetic testing to determine that patients are homozygous or compound heterozygous for pathogenic *PYGM* mutations.<sup>3</sup> This is an important consideration because recent research using next-generation sequencing of the *PYGM* gene indicates that many patients remain undiagnosed and that current estimates of disease prevalence, e.g., ~1/167,000 in Spain,<sup>3</sup> are probably too low.<sup>5</sup> Thus, efforts are needed to facilitate efficient diagnosis of the disease. Furthermore, timely and accurate recognition of this disorder might result in patients’ adopting lifestyle habits that have a documented therapeutic effect as early as possible,<sup>4</sup> such as following a diet rich in complex carbohydrates and performing regular physical activity of moderate intensity while avoiding potentially harmful exercises (e.g., isometric

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exercises) that increase the risk of rhabdomyolysis and its deleterious consequences.<sup>6,7</sup>

A total of 148 mutations in the *PYGM* gene have been reported to cause McArdle disease.<sup>8,9</sup> Sanger sequencing, currently the most commonly used method in diagnostic reports, can not only fail to detect some mutations in a timely matter but also (and even more importantly) fail to unveil the actual pathogenicity of these mutations.<sup>10</sup> Thus, further studies might be needed with muscle cDNA.<sup>11</sup> In addition to the invasive nature of the muscle biopsy procedure, the usefulness of analyzing cDNA synthesized from tissue-specific transcripts is limited by a cellular homeostatic mechanism known as “nonsense-mediated mRNA decay” (NMD), which eliminates aberrant transcripts that contain nonsense and frameshift mutations.<sup>12,13</sup> Previous data from our group have indicated that tissue-specific NMD is highly prevalent (>90%) in McArdle disease, including the commonest *PYGM* mutation among Caucasians, p.R50X (or p.R50\*<sup>14</sup>).

There is some residual transcription of tissue-specific genes in virtually all body cells, a phenomenon originally described as “illegitimate”<sup>15</sup> or “ectopic” transcription.<sup>16</sup> By studying muscle-type dystrophin messenger RNA (mRNA) in easily accessible cells (e.g., peripheral blood mononuclear cells (PBMCs)) from patients with Duchenne and Becker muscular dystrophies caused by a known internal gene deletion, Chelly et al.<sup>17</sup> demonstrated the similarity of tissue-specific transcripts with their “illegitimate” surrogates; thus, the latter were considered a valuable tool with which to study gene defects at the mRNA level. Furthermore, the authors’ data suggest that NMD does not operate in nonmuscle cells. As such, it would theoretically be possible to use these cells for studying the actual pathogenicity of mutations that are affected at the specific-tissue level by NMD, particularly silent or splicing mutations.

In the present study, we obtained *PYGM* transcripts in easily accessible cells (i.e., PBMCs) as well as in muscle biopsy specimens, from McArdle patients and healthy controls with two purposes, diagnostic and pathobiological: to determine (i) whether PBMCs are a valid source of *PYGM* mRNA that enables detection of *PYGM* mutations and, in particular, (ii) whether studies of PBMCs are more suitable than muscle analyses to gain insight into the actual functional consequences and pathogenicity of NMD-affected mutations.

## MATERIALS AND METHODS

### Patients and controls

Written consent was obtained from all participants. The study was approved by the institutional ethics committee (Hospital Universitario 12 de Octubre, Madrid, Spain) and was in accordance with the Declaration of Helsinki for Human Research. We studied 12 patients with McArdle disease (10 male, 2 female, age range 14–59 years) from 10 different families (patients 5 and 6 and patients 11 and 12 were siblings). They were selected because (i) they had the two most prevalent *PYGM* mutations in the Spanish population (i.e., p.R50\* and p.W798R), which, accordingly, are the ones first looked for with direct genomic

**Table 1** *PYGM* mutation profile in the study patients

Patient	Mutation in each allele	Type of mutation	Mutation detected in PBMC cDNA?	Mutation detected in muscle cDNA?
P1	p.G205S/	Missense	Yes	Yes
	<b>c.529-8G&gt;A</b>	<b>Splicing</b>	Yes	Yes
P2	p.R50*/	Nonsense	Yes	No
	<b>c.1970_2177del</b>	<b>Deletion</b>	Yes	Yes
P3	<b>c.645G&gt;A/</b>	<b>Silent</b>	Yes	No
	p.R50*	Nonsense	Yes	No
P4	p.R50*/	Nonsense	Yes	N.A.
	p.R50*	Nonsense	Yes	N.A.
P5	<b>c.645G&gt;A/</b>	<b>Silent</b>	Yes	No
	p.W798R	Missense	Yes	Yes
P6	<b>c.645G&gt;A/</b>	<b>Silent</b>	Yes	No
	p.W798R	Missense	Yes	Yes
P7	p.W798R/	Missense	Yes	Yes
	p.W798R	Missense	Yes	Yes
P8	p.R50*/	Nonsense	Yes	No
	p.W798R	Missense	Yes	Yes
P9	p.R50*/	Nonsense	Yes	N.A.
	p.R50*	Nonsense	Yes	N.A.
P10	p.R50*/	Nonsense	Yes	N.A.
	p.L587P	Missense	Yes	N.A.
P11	p.G205S/	Missense	Yes	N.A.
	<b>c.1768 + 1G&gt;A</b>	<b>Splicing</b>	Yes	N.A.
P12	p.G205S/	Missense	Yes	N.A.
	<b>c.1768 + 1G&gt;A</b>	<b>Splicing</b>	Yes	N.A.

Detection (or not) of mutations in cDNA synthesized from muscle or peripheral blood mononuclear cells (PBMCs) is indicated. Genbank reference sequences used to label *PYGM* mutations are NM\_005609.2 and NP\_005600.1. Mutations that are difficult to detect at the direct genomic DNA analysis and that usually require cDNA studies in muscle biopsies are shown in bold type.

cDNA, complementary DNA; NA, (sample) not available.

analyses,<sup>3</sup> or (ii) they harbored types of mutations (i.e., large deletions, silent or splicing mutations) whose pathogenicity is difficult to identify with DNA Sanger sequencing and thus require further studies (with cDNA) in muscle biopsy samples (Table 1). All patients showed the typical features of the disease, i.e., exercise intolerance since childhood with some episodes leading to severe hyper-CK-emia (ranging from 2,300 to 15,000 U/l; normal levels <170 U/l). All patients had experienced at least one episode of myoglobinuria.

The *PYGM* genotype of patients P1–9 has been reported in previous studies,<sup>11,14</sup> whereas the *PYGM* genotype of patients P10–12 was determined here for the first time (Table 1). Blood samples were obtained from all 12 patients and muscle samples were obtained from 7 patients. We also studied 7 muscle biopsy samples and 14 blood samples, all of which corresponded to healthy individuals with no history of exercise intolerance, primary muscle disease, or major chronic condition.

### DNA/RNA isolation

PBMCs were isolated from whole blood (EDTA tubes) of patients and healthy controls by Ficoll-Hypaque gradient

centrifugation according to the manufacturer's instructions (Amersham, Buckinghamshire, UK). Genomic DNA and total RNA were obtained from PBMCs using Tripure Isolation Reagent (Roche Molecular Biochemicals, Indianapolis, IN). Total RNA was extracted from the skeletal muscle samples using Totally RNA (Ambion, Austin, TX) and treated with amplification-grade deoxyribonuclease I (Invitrogen, Carlsbad, CA) to eliminate any traces of DNA. Nucleic acid concentration and quality were analyzed with NanoChips using the Bioanalyzer 2100 system (Agilent, Santa Clara, CA).

#### Separation of PBMC into CD14<sup>+</sup> and CD14<sup>-</sup> cells

PBMCs (~10<sup>7</sup> cells) were mixed with 20  $\mu$ l of CD14-coated microbeads (Milteny Biotec, Bergisch Gladbach, Germany) and incubated to 4°C for 15 minutes. Unbound microbeads were removed by washing cells in excess phosphate-buffered saline buffer, followed by centrifugation at 300g for 10 minutes. The cell pellet was resuspended in phosphate-buffered saline buffer with a concentration of 2  $\times$  10<sup>8</sup> cells/ml before separation on an apparatus for magnetic cell separation according to the manufacturer's instructions (MACS; Milteny Biotec). The purity of the CD14<sup>+</sup> cell population was checked by fluorescence-activated cell sorting and exceeded 95%. Total RNA was isolated from CD14<sup>+</sup> and CD14<sup>-</sup> cell populations using Tripure Isolation Reagent (Roche Molecular Biochemicals).

#### cDNA synthesis

The cDNAs of PBMCs and CD14<sup>+</sup> and CD14<sup>-</sup> cell populations were synthesized using the Transcriptor First Strand cDNA Synthesis Kit from Roche Applied Science. The 20 exons of *PYGM* cDNA were amplified in six overlapping fragments. Primers and polymerase chain reaction (PCR) conditions are detailed in **Supplementary Table S1** online. Skeletal muscle cDNA was synthesized using the high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Muscle *PYGM* cDNA was amplified by PCR in two overlapping fragments as previously reported.<sup>11</sup> Control amplification was tested by cDNA amplification of a 1,200-bp fragment of the porphobilinogen deaminase (*PBGD*) "housekeeping" gene.

#### Molecular genetic analysis

Both amplified genomic DNA and cDNA underwent Sanger sequencing, as described elsewhere,<sup>14</sup> using the ABI 3100XL genetic analyzer (Applied Biosystems). Because these samples were biological replicates, we performed this experiment only once. The Genbank reference sequences for protein, NP\_005600.1, and for cDNA, NM\_005609.2, were used.

PCR conditions were 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing for 30 seconds, and extension at 72°C for 1 minute and 30 seconds. Initial denaturation at 94°C for 5 minutes and a final extension at 72°C for 10 minutes were performed. Each 50- $\mu$ l reaction contained 1.0 U *Taq* polymerase (Bioline USA, Randolph, MA), 20 pmol of each primer,

200  $\mu$ mol/l of deoxynucleotides, 1.5 mmol/l MgCl<sub>2</sub> in the buffer supplied, and 0.5  $\mu$ l of cDNA.

#### Real-time PCR

*PYGM* mRNA levels were quantitated by real-time PCR using TaqMan fluorogenic probes and a 7500 Real-Time PCR System (Applied Biosystems) as described elsewhere.<sup>14</sup> All reactions were performed in triplicate and analyzed using the Applied Biosystems SDS 7500 system software (Applied Biosystems). Expression values were recalculated considering 100% as the average of the results obtained in the skeletal muscle control sample 1.

#### Immunoblot analysis

PBMC proteins were extracted as previously described.<sup>18</sup> Skeletal muscle samples were homogenized in five volumes of phosphate-buffered saline buffer (20 mmol/l sodium phosphate/0.5 mol/l NaCl, pH 7.4) containing 1% sodium dodecyl sulfate, and the homogenate was centrifuged at 14,000 rpm for 1 minute. The supernatant was carefully transferred (soluble protein fraction) to a clean tube.

Protein concentrations were measured using a modified Bradford assay, and samples were diluted in a sample buffer (250 mmol/l Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 10% glycerol, 2%  $\beta$ -mercaptoethanol, and 0.002% bromophenol blue) and boiled for another 5 minutes. Samples were separated on a sodium dodecyl sulfate-7% polyacrylamide gel, and the proteins were electrophoretically transferred to a polyvinylidene difluoride filter at 50 V for 1 hour in a transfer buffer containing 20 mmol/l Tris, 150 mmol/l glycine, and 10% methanol. The filter was then blocked in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% dried milk powder (weight/volume) overnight at 4°C. After three washes with TBST, the polyvinylidene difluoride filters were incubated with primary antibody to myophosphorylase (ref. ab63158; Abcam, Cambridge, UK) for 1 hour at room temperature. After another three washes with TBST, the filters were incubated with a goat antirabbit peroxidase-conjugated immunoglobulin G (Molecular Probes, Invitrogen) for 1 hour at room temperature and then washed for 30 minutes with TBST.

Immunoreactive proteins were visualized using the enhanced chemiluminescence western blotting detection system (Amersham). The light-emitting bands were detected with X-ray film. The resulting band intensities were quantitated using ImageJ 1.38 (National Institutes of Health, Bethesda, MD); to control sampling errors, we determined the ratios of band intensities to  $\alpha$ -tubulin (Sigma, St. Louis, MO) in skeletal muscle samples, and  $\beta$ -actin (Sigma) in PBMCs. Immunoblot analysis was performed at least twice for each sample.

## RESULTS

We performed RNA extraction in a total of 14 muscle biopsy specimens (corresponding to 7 patients and 7 controls) and 26 blood samples (from 12 patients and 14 controls). The

procedures used for RNA extraction and cDNA synthesis in muscle and PBMCs are illustrated in **Supplementary Figure S1** online.

### Muscle cDNA: confirmation of NMD occurrence

The sequences of cDNA synthesized from muscle transcripts showed the profile expected for point mutations belonging to the group of silent intronic mutations, as explained below. First, the splicing mutation c.529-8G>A produced an insertion of two codons codifying for valine and glutamine in positions 176 and 177, respectively, thereby resulting in a myophosphorylase molecule with two extra amino acids, whereas the large deletion c.1970\_2177del resulted in the total absence of exon 17. However, transcripts with the silent mutation c.645G>A (p.K215=, found in P3, P5, and P6) were not found in muscle cDNA.

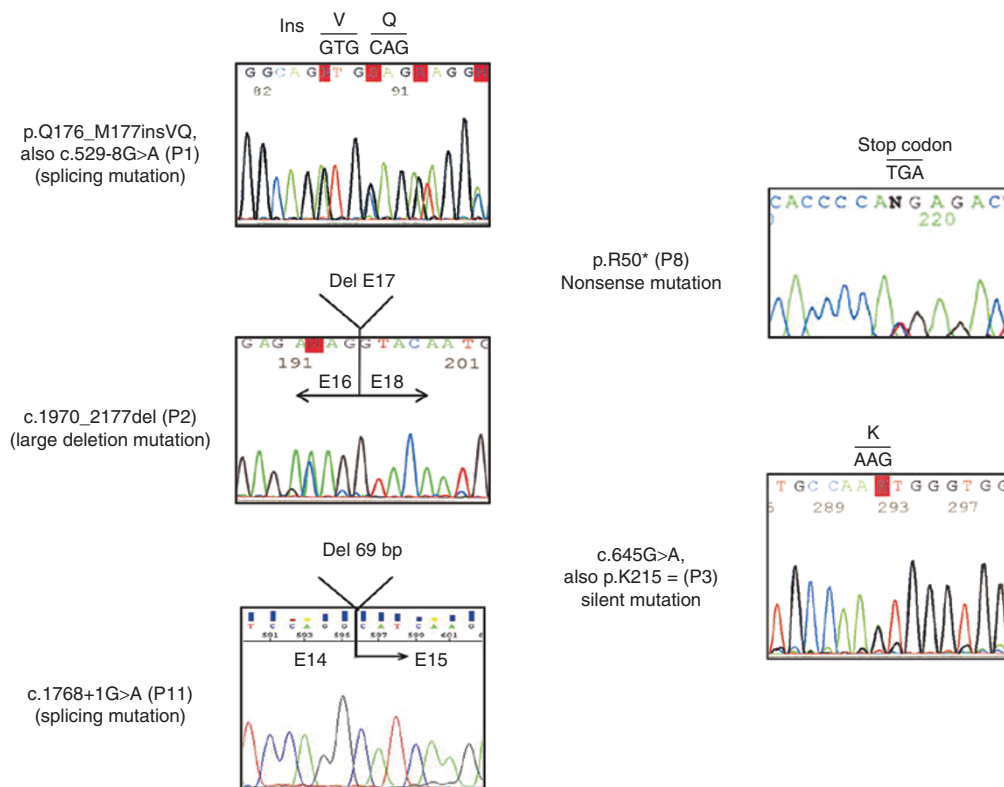
However, we could not find the sequences of the commonest nonsense mutation p.R50\* in the muscle cDNA of any of the six patients harboring this variant (P2-4, P8-10), which is in line with previous research showing that this mutation consistently undergoes NMD.<sup>14</sup> By contrast, the missense mutations (p.G205S, p.W798R, p.L587P) were detectable, which is also in agreement with previous research indicating that missense mutations in the *PYGM* gene do not appear to be affected by NMD.<sup>14</sup>

### PBMC cDNA: no evidence of NMD

To determine whether there were differences in RNA extraction or in the detected gene sequences among different PBMC subpopulations, we compared total PBMCs with CD14<sup>+</sup> and CD14<sup>-</sup> lymphocytes and found no differences. Therefore, all the subsequent (and herein reported) analyses were performed using total PBMCs.

Importantly, and in contrast to muscle analyses, all the studied mutations were certainly detectable in PBMCs. First, mutations with unknown effects such as silent or splicing mutations and that might require cDNA studies at the specific tissue level to be characterized were detected in PBMCs (**Figure 1**, left panel). This was also the case of the splicing mutation c.1768+1G>A, which was found in the PBMCs from patients P11 and P12. This variant causes a deletion of 69bp in the 3' terminal region of exon 14, resulting in a mutation that does not alter frameshift. Although no biopsy samples were available from these two patients, preservation of the open reading frame explains why this mutation has also been detected at the muscle-tissue level in previous research.<sup>19</sup>

In fact, PBMCs were more suitable for unveiling the actual effects of some *PYGM* mutations compared with muscle biopsy samples. As opposed to muscle tissue, the NMD did not seem to operate in these cells, as shown for the c.645G>A (or p.K215=) and for the p.R50\* mutation (**Figure 1**, right panel). Notably, the



**Figure 1** Electropherograms of cDNA synthesized from mRNA in peripheral blood mononuclear cells (PBMC transcript analysis). Five mutations were identifiable: (i) those that are not easily detectable with direct genomic DNA analysis, and thus require muscle cDNA analysis (left panel); and (ii) those that are not detectable at the cDNA muscle level due to elimination of mRNA by the nonsense-mediated decay mechanism (NMD) (right panel). A, adenine; C, cytosine; cDNA, complementary DNA; E, exon; G, guanine; Ins, insertion; K, lysine; mRNA, messenger RNA; P, patient; Q, glutamine; T, thymine; V, valine.

c.645G>A mutation showed the inclusion of the full intron 6 in the blood transcripts with subsequent addition of 91 bp and an altered reading pattern (Figure 2). This transcriptomic alteration was unnoticed in previous research reporting the c.645G>A for the first time and using muscle cDNA<sup>11</sup> because the NMD mechanism acts at the specific tissue level<sup>11</sup> but not in PBMCs.

**PYGM transcript and myophosphorylase levels in muscle and PBMCs**

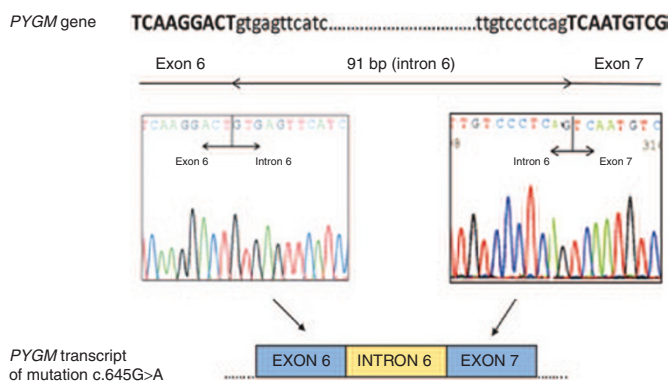
We compared the amount of PYGM transcripts and myophosphorylase protein between PBMCs and muscle tissue using real-time PCR and western blot analyses, respectively. The mean PYGM transcription level in PBMCs was less than 0.01% of the average value in muscle, a finding that was corroborated in patients and healthy controls (Mann–Whitney *U*-test, *P* < 0.001 in both; Figure 3, left panel). In the western blot analyses, and after loading 50 µg of protein of each sample, myophosphorylase protein was undetectable in PBMCs from healthy controls,

whereas gene-product expression was abundant in the healthy muscle tissue (Figure 3, right panel).

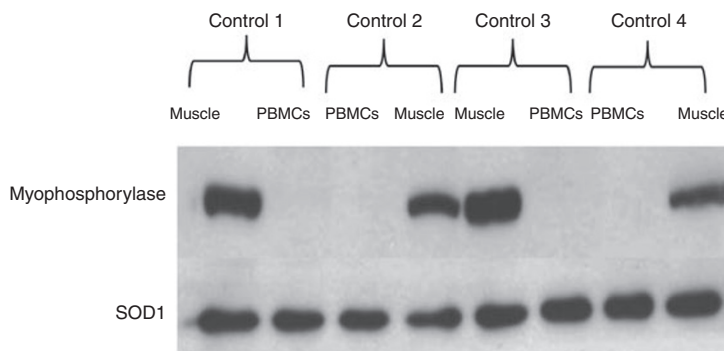
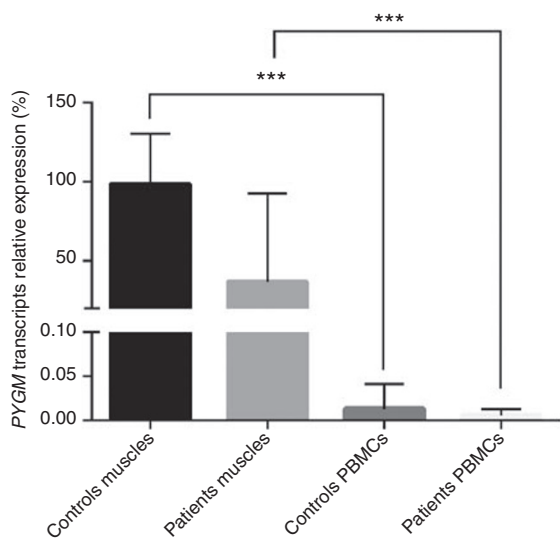
**DISCUSSION**

We have reported a simple, novel method of transcriptomic analysis for facilitating the diagnosis of a “pure myopathy” (i.e., whose molecular defect is confined to the muscle tissue), McArdle disease, using only blood samples with no need for muscle biopsies. We assessed a representative subset of the main types of mutations (missense, nonsense, deletion, silent, or splicing mutations) in PBMCs from McArdle patients. The main findings of our study were twofold. First, mutations whose actual pathogenic effects are unknown (silent or splicing mutations) could be characterized in PBMCs. Second, the use of muscle biopsies for cDNA analysis does not offer an advantage over the less invasive approach proposed here. In fact, because the protective NMD mechanism (aiming at preventing excessive accumulation of aberrant transcripts) does not seem to operate in nonspecific cells showing only residual gene transcription, PBMCs are actually more suitable for analyzing the effects of PYGM mutations compared with muscle biopsy samples.

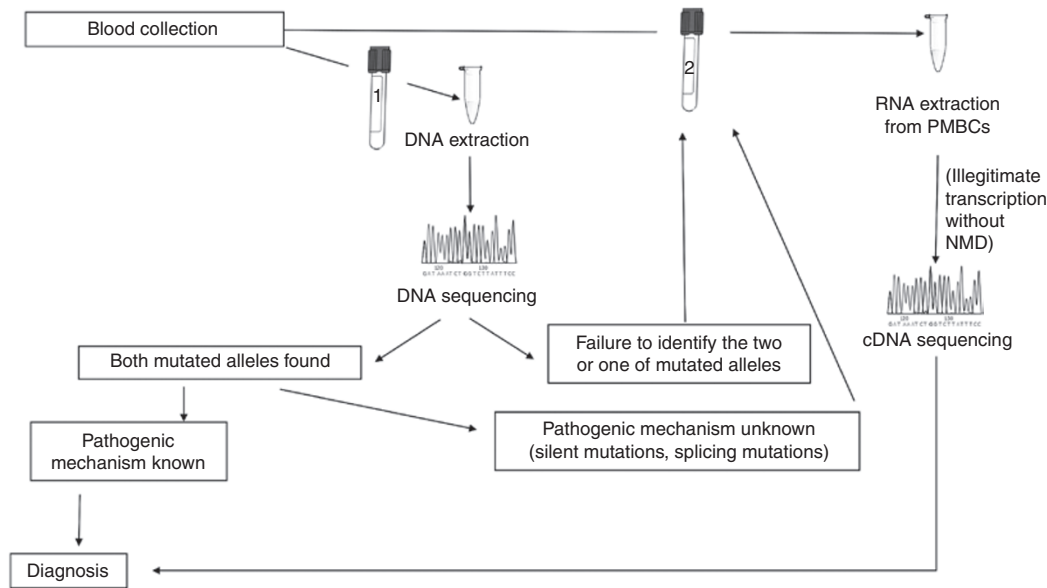
We believe that our results are of potential applicability and suggest that collection of just two blood tubes—one for direct genomic analysis and one for transcript analysis of PBMCs in case the first approach fails—suffices for ensuring full and efficient diagnosis of McArdle disease (Figure 4). It should be emphasized that, because the method presented here does not involve the cloning step, the transcripts observed in DNA sequences will be the most represented ones in the PCR pool. We believe that, for the purpose of assessing the pathogenicity of a mutation with an unknown effect, characterization of the main transcript should normally suffice to provide information on the pathological consequences at the mRNA level. Nevertheless, cloning and sequencing of the PCR fragments is



**Figure 2 Transcript alteration associated with the mutation c.645G>A studied in peripheral blood mononuclear cells (PBMCs).** Two electropherograms flanking the insertion of full intron 6 in the PBMC transcripts are shown. A, adenine; C, cytosine; G, guanine, T, thymine.



**Figure 3 Real-time polymerase chain reaction of PYGM transcripts in patients and controls (left panel) and western blot myophosphorylase in controls (right panel).** Values are represented by mean, and error bars represent 2 SD. PBMC, peripheral blood mononuclear cell; SOD1, superoxide dismutase 1. \*\*\**P* < 0.001.



**Figure 4 New strategy proposed for McArdle diagnosis.** Collection of only two blood EDTA tubes might suffice for the diagnosis. The first tube is used for DNA extraction and direct *PYGM* gene sequencing. If one or two mutant alleles are not identified or if mutations with an unknown pathological effect are detected, then RNA is extracted from the peripheral blood mononuclear cells (PBMCs) in the second EDTA tube. Thereafter, the methodology outlined in this article can be used for cDNA sequencing, which allows efficient mutation detection as well as an understanding of the mechanisms responsible for eventual pathogenicity. cDNA, complementary DNA; NMD, nonsense-mediated mRNA decay.

obviously still necessary to determine the presence of multiple transcript variants. In this regard, an additional potential application of the method proposed here is that it allows characterization of alternative gene splicing patterns.

The facts that mutations have been reported in each of the 20 *PYGM* exons and there is probably no evident “hot spot region”<sup>20</sup> can make direct genomic analysis (e.g., with the commonly reported Sanger method) time-consuming. Failure to identify one or two mutant *PYGM* alleles might occur in ~24% of cases when following a defined and sequential protocol for DNA diagnosis based on Sanger sequencing.<sup>10</sup> This, together with the large allelic heterogeneity and growing number of *PYGM* genotypes that cause McArdle disease (with most mutations being private, i.e., reported in a single patient or in members of the same family), suggests that numerous pathogenic variants remain to be discovered.<sup>8</sup>

In addition to its methodological advantages in terms of easing diagnosis, transcriptomic analysis of PBMCs might help provide insight into the pathogenicity of *PYGM* variants, as exemplified by the study of the silent mutation c.645G>A (p.K215=). Interestingly, the Exome Aggregation Consortium (<http://exac.broadinstitute.org>), a data set of 60,706 unrelated individuals whose DNA was sequenced as part of disease-specific and population genetic studies, has reported a frequency of 0.0044 and the existence of three homozygous individuals for this predicted synonymous variant (rs116315896), thus stirring some controversy about its actual pathogenicity. However, our transcriptional studies of patients’ blood samples show that this mutation impairs the correct processing of intron 6 splicing, thereby producing the full inclusion of this intron in the mature

transcript. Hence, it alters the *PYGM* transcript open reading frame because of the inclusion of 91 bp and thus activates the NMD mechanism. The functional consequences found here could not be identified in previous transcriptomic studies involving muscle biopsy specimens from McArdle patients because the NMD operates in this tissue.<sup>11</sup>

The following findings support the pathogenicity of the p.K215= mutation: the three McArdle patients in our study who harbored this variant (Table 1) showed absence of muscle myophosphorylase stain; they were compound heterozygotes carrying other well-known pathogenic mutations, i.e., p.R50\* or p.W798R; and no other variants were found after sequencing the entire *PYGM* coding region as well as intron/exon boundaries. Additional supporting points are that (i) no additional mutations inside intron 6 (which is included in the mRNA sequence of these patients) have been found to date; (ii) the p.K215= mutation is located five nucleotides upstream the splicing donor site of intron 6 and is thus the only genetic change detected in the surrounding area of the splicing alteration; and (iii) a recent timely publication by De Castro et al.<sup>5</sup> estimated that ~75% of North American McArdle patients remain undiagnosed and therefore some of the unidentified patients may be homozygous according to the Exome Aggregation Consortium. Nevertheless, we cannot disregard the fact that the p.K215= mutation might be in linkage disequilibrium with the real pathogenic variant, which can ultimately be corroborated only by using an *in vitro* model for this mutation.

For those involved in research on McArdle disease or in the management of patients, it might be useful to know that, at least for the time being, the p.K215= variation mutation should

be considered pathogenic. However, it is the first mutation in the *PYGM* gene whose effects on the splicing mechanisms were discovered through the novel method reported here. This approach could also be used to determine the actual functional consequences of other silent mutations and most intronic mutations that are known to cause the clinical phenotype that characterizes McArdle disease.<sup>8</sup> In fact, another strength of the method reported here is that it could be an alternative to costly and time-consuming animal functional models or *in silico* models, which are often difficult to interpret.<sup>21</sup>

Residual transcription of tissue-specific transcripts (e.g., *PYGM* mRNA) in PBMCs reflects a phenomenon originally termed “illegitimate transcription,” which is thought to occur in virtually all cells<sup>15</sup> and has been demonstrated in the lymphoid cells of patients with two other neuromuscular disorders: Duchenne and Becker dystrophies.<sup>17</sup> Not all cell types are necessarily suitable for adequate characterization of muscle-specific transcripts. This is the case of other types of neuromuscular disorders, such as dysferlinopathies, in which isolation of peripheral monocytes is needed to characterize *DYSF* gene transcripts.<sup>18,22,23</sup> However, no differences were found here between CD14<sup>+</sup> isolated cells and the total PBMC population, indicating that residual *PYGM* transcription occurs similarly in different blood-cell populations.

Despite the occurrence of some level of transcription, we failed to detect the final *PYGM* gene end product, myophosphorylase, in PBMCs, even after loading the western blots with high amounts (50 µg) of protein. This might indicate that the western blot technique is not sensitive enough for detecting myophosphorylase, especially considering that recent research using flow cytometry showed that T lymphocytes can express myophosphorylase.<sup>24</sup> Taken together, the present data and those from previous research with flow cytometry<sup>24</sup> suggest that the total amount of myophosphorylase expressed in blood cells is very low, even in healthy people, which is consistent with the finding that 13% of McArdle patients showed the same percentage of myophosphorylase-positive white blood cells as that in healthy controls.<sup>24</sup> In this regard, *in vitro* cell treatment with cycloheximide can increase the abundance of nonspecific transcripts and therefore of the protein.<sup>25</sup> Thus, more studies are needed to elucidate the effect of cycloheximide treatment on myophosphorylase expression in blood cells. More importantly, more research is needed to answer these fundamental questions: why do nonspecific tissues express some amount of gene-specific transcripts, such as the one coding myophosphorylase, and do these residual amounts of transcript play an actual biological role? In-depth studies of “illegitimate” transcripts might help to answer these questions. For instance, myophosphorylase might not be involved only in muscle glycogen metabolism; recent data suggest that it might also play a role in the regulation of lymphocyte-T-cell proliferation.<sup>26</sup>

In summary, a main advantage of the transcriptomic analysis of PBMCs is that it might help in efforts to gain insight into the pathogenicity of *PYGM* variants with no need for studying muscle biopsy specimens. This was exemplified by the study of

the silent mutation c.645G>A, whose effect at the transcription level was previously unknown. Furthermore, the upcoming use of next-generation sequencing–targeted gene panels or whole-exome/whole-genome sequencing in the neuromuscular clinic framework will presumably result in identification of several novel *PYGM* mutations whose actual pathogenicity would have to be corroborated with a method such as the one proposed here.

## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/gim>

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## DISCLOSURE

The authors declare no conflict of interest.

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